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Long-Term Culture of Decapsulated Gastropod Embryos: A Transplantation Study

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Abstract. Encapsulated embryos of the pond snail *Helisoma trivolvis* have been useful for examining neural development and neural circuit function during development. However, their full potential in developmental studies is limited by the lack of an effective method for long-term culture of decapsulated embryos. In the present study, decapsulated early embryos were either cultivated *ex ovo* in various media under different environmental conditions or transplanted into host egg capsules. Although diluted capsular fluid, 30% M199, and albumen-gland-conditioned medium were partially effective in promoting embryonic growth for a short time, none of the media promoted normal embryonic development in long-term tests. In contrast, after previously decapsulated and experimentally manipulated embryos were transplanted into host capsules, their growth and development were similar to their intact siblings. In combination with laser ablation, this transplantation technique was used to demonstrate the role played by a pair of serotonergic neurons in regulating an embryonic rotational behavior. These results suggest that embryonic transplantation is an extremely effective technique for achieving long-term growth and development of previously decapsulated embryos and therefore can be instrumental in investigating cell lineage, function, and development in encapsulated embryos.

Introduction

Embryos of pulmonate gastropods undergo direct development from fertilized egg to juvenile snail inside egg capsules filled with albuminous perivitelline fluid, also known as capsular fluid (CF). The nutrition required for normal development of embryos is provided by the CF, which is a secretory product of the albumen gland (Raven, 1958; Morrill *et al.*, 1964; Beadle, 1969; Goudsmit, 1976; Morrill, 1982; Stockmann-Bosback and Althoff, 1989; Heras *et al.*, 1998). Early studies identified carbohydrates such as galactogen and proteins as two major categories of nutrition in CF, which together make up more than 94% of its dry weight in the freshwater pond snail *Lymnaea stagnalis* (Horstmann, 1956; Morrill *et al.*, 1964; Wijsman and van Wijck-Batenburg, 1987). However, the specific key nutritive components for the growth and development of early embryos are yet unknown. In addition, several other unique features of the microenvironment of egg capsules can hardly be mimicked *in vitro*. Among such features are a positive hydrostatic pressure (2.1 atmospheres) and negative equilibrium potential (−23 mV) inside newly laid egg capsules of *Lymnaea* (Raven, 1958; Taylor, 1973; Morrill, 1982; Pechenik *et al.*, 1984); antibacterial factors in the CF of the sea slug *Aplysia kurodi* (Kamiya *et al.*, 1984); and an epidermal growth factor (EGF) and various enzymes such as phosphatases and trypsin inhibitors in the CF of several species (Stockmann-Bosback and Althoff, 1989; Hermann *et al.*, 2000; Nagle *et al.*, 2001). These complex structural and compositional features of egg capsules appear to be critical for embryonic development, as attempts to achieve normal development of early stage embryos *ex ovo* have been largely unsuccessful in many species (Morrill, 1982; Pechenik *et al.*, 1984; Stockmann-Bosback and Althoff, 1989; Meshcheryakov, 1990).

In experiments that involve perturbation of embryonic development and function, embryos must be isolated from their egg capsules for experimental treatments and then monitored as they continue to develop. For example, embryos of several encapsulated gastropods display a cilia-driven rotational behavior early in their development (Mor-

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Abbreviations: AHS, antibiotic *Helisoma* saline; APW, artificial pond water; CF, capsular fluid; EGF, epidermal growth factor; HDM, *Helisoma* defined medium.

rill, 1982; Diefenbach *et al.*, 1991; Voronezhskaya *et al.*, 1999). In *Helisoma trivolvis*, the occurrence of this rotation is accompanied by the differentiation of a pair of embryonic serotonergic neurons identified as ENC1s (Goldberg and Kater, 1989; Diefenbach *et al.*, 1998; Koss *et al.*, 2002). In acute experiments on isolated embryos, ENC1s have been shown to be cilioexcitatory motor neurons that accelerate embryonic rotation in response to hypoxia (Kuang and Goldberg, 2001; Kuang *et al.*, 2002). To assess whether this response is an adaptation that affects embryonic viability and recruitment success, and to examine the mechanisms regulating development of the ENC1-ciliary neural circuit, we need methods that promote normal embryonic development after embryos have been decapsulated and treated.

The goal of this study was to generate a method for long-term culture of decapsulated *Helisoma* embryos. We first tested the effects of pH, epidermal growth factor, and various culture media on the *ex ovo* growth and development of decapsulated embryos. We then tested the feasibility of transplanting previously decapsulated embryos into egg capsules containing host embryos for long-term culture. Our results indicate that transplantation is an effective method for continued growth and development of previously decapsulated embryos. This transplantation technique will facilitate studies of embryonic cell function, lineage, and development of encapsulated species.

Materials and Methods

Animals

Adult individuals of *Helisoma trivolvis* were maintained in flow-through aquaria containing dechlorinated water (25 °C) and were fed with lettuce and Trout Chow (Unifeed). Since the pond snails usually lay eggs on a smooth surface, plastic petri dishes (diameter 150 mm) were placed in the aquaria to facilitate egg-laying. Egg masses were collected from the petri dishes with a razor blade and transferred into artificial pond water (APW: 0.025% Instant Ocean, pH 7.2–7.3, Aquarium Systems) for experimental use. Embryos were staged as the percentage of the whole intracapsular development, with E0 corresponding to the zygote at 0% development and E100 corresponding to hatching at 100% development (Goldberg *et al.*, 1988; Goldberg, 1995; Diefenbach *et al.*, 1998). In this study, egg masses containing stage E0 to E25 embryos were used.

Ex ovo *culture of isolated embryos*

Clean and intact egg masses containing embryos of appropriate stages were washed three times, for 30 min each time, in antibiotic APW (150 μg/ml Gentamicin) to disinfect the surface of the egg mass. Embryos were then isolated in antibiotic *Helisoma* saline (AHS: 51.3 m*M* NaCl, 1.7 m*M* KCl, 4.1 m*M* $CaCl_2$, 1.5 m*M* $MgCl_2$, 5.0 m*M* HEPES, 150 μg/ml Gentamicin, pH 7.30–7.35, 115–119 mOsm) under sterile conditions. The egg mass and capsular membranes were ruptured with a 30-gauge surgical needle, and the embryos were gently blown or sucked out with a refined and heat-polished Pasteur pipette.

The isolated embryos were then cultured at 23 °C in 2 ml of various media in 35-mm petri dishes (Falcon 3001). The media tested included AHS; antibiotic APW; *Helisoma* defined medium (HDM: 50% Leibovitz-15 (Gibco), 40.0 m*M* NaCl, 1.7 m*M* KCl, 4.1 m*M* $CaCl_2$, 1.5 m*M* $MgCl_2$, 5.0 m*M* HEPES, 50 μg/ml Gentamicin, 150 μg/ml l-glutamine, pH 7.30–7.40, 143 mOsm); 30% M199 (Gibco, pH 7.20–7.30, 91 mOsm); 5% galactose in AHS; 1.5% CF in AHS; and albumen-gland-conditioned AHS. The 1.5% CF was harvested by releasing all intracapsular contents from 60 egg capsules into 2 ml AHS with a pipette and subsequently removing the embryos. The average volume of egg capsules was estimated to be 0.47 μl on the basis of their nearly oval shape and maximal height (916 ± 49 μm, n = 7) and length (1066 ± 16 μm, n = 26). The albumen-gland-conditioned medium was made by incubating isolated albumen glands in AHS (2 glands/ml, 2–12 h at 23 °C) in the presence of 100 μ*M* forskolin, a cyclic AMP activator previously shown to stimulate release of proteins and polysaccharides from the albumen gland (Morishita *et al.*, 1998).

To mimic the capsular environment, isolated embryos were cultured separately in hanging droplets of culture media and in culture media with increased viscosity (1% gelatin). To test the effect of pH on growth and development, isolated embryos were cultured in HDM of different pH (6.4, 7.4, and 8.4). To test the effect of epidermal growth factors (EGF) on growth and development, isolated embryos were cultured in HDM plus 0.1 μg/ml human recombinant EGF (h-EGF, Sigma), or in a novel EGF purified from the CF of *Lymnaea* (l-EGF, a generous gift from Dr. A. Bulloch, Univ. of Calgary).

The cultured embryos were viewed with an inverted microscope (Zeiss Axiovert) and the digital images were captured by a CCD video camera (Paultek Imaging). The length (maximal dimension) of the embryos was analyzed with NIH image (http://rsb.info.nih.gov/nih-image/).

Transplantation of early embryos

Before they were transplanted into new host capsules, isolated embryos were washed twice in AHS and then either placed in AHS for at least 1 h or experimentally manipulated. Embryos removed from their egg capsules for as long as 2 h before transplantation developed normally after transplantation, allowing sufficient time to perform experimental manipulations. Longer times were not tested. In transplantation of stage E15–E25 embryos, the host egg capsules contained younger embryos (≤stage E10) so that host and transplanted embryos could easily be distinguished. For

transplantation of embryos at the 2-cell to 8-cell stages (E1–E2), isolated embryos were transplanted into capsules whose host embryos had been killed by heat shock (water bath at 48 °C for 25 min).

Prior to transplantation, the host egg mass was stabilized at the center of a 35-mm petri dish (Falcon 1008) by surrounding the mass with a strip of dental wax. The host egg mass was then immersed in filtered APW. Transplantation was performed through a 150–300 μm incision made near the edge of each host capsule. In each donor egg mass, half of the embryos were isolated for transplantation; the remaining embryos were kept as *in ovo* controls. A Sigmacote (Sigma)-coated, heat-polished glass micropipette (tip diameter: 120–250 μm depending on the stage of embryos to be transplanted) was used to transfer the embryos. The micropipette was operated by hand and pressure control was exerted with a 0.2-ml calibrated micrometer syringe (Gilmont). The transplanted and control embryos were viewed with an inverted microscope (Nikon Eclipse TE300), and digital images were captured by a CCD camera (Cooke SensiCam). The maximal length of the embryos was analyzed with image analysis software (Slidebook, Intelligent Imaging Innovations, Inc., Denver, CO).

Laser ablation of embryonic cells

The standard procedures for laser ablation are described elsewhere (Kuang and Goldberg, 2001). Briefly, embryos were isolated, mounted on slides, and viewed with differential interference contrast (DIC) optics. Only those with both ENC1s clearly visible were used (about 10% of stage E25 embryos). Pulsed laser beams (pulse length: 3 ns) generated by a VSL-337 nitrogen laser (Laser Science, Inc.) were delivered to the targeted cells *via* a coupling unit (Photonic Instruments, Inc.) and the optical path through a compound microscope (Zeiss, Axioskop). The coupling unit contained a dye laser module (5 m*M* coumarin 440), emitting at a wavelength of 420–475 nm. Laser beams, which theoretically had a diameter equal to the wavelength, were attenuated to a strength that was just able to scratch glass coverslips and were applied at a frequency of 8–10 Hz onto the nucleolus and perinucleolar region of the nuclei. To kill a cell, 200–300 pulses were applied until visible scars or blebs were apparent on the nucleolus, the nuclear boundary became obscure, and the cell started to swell. This procedure induced a gradual cell death that was complete by 6–24 h after laser treatment (Kuang and Goldberg, 2001).

Measurement of embryonic rotation

To test the role of ENC1s in regulating embryonic rotation, stage E25 embryos with ENC1s ablated were transplanted into new egg capsules and embryonic rotation was monitored the following day with a CCD video camera (JVC, TK860U) mounted on a dissecting microscope (Zeiss, StemiSR). Rotation was recorded at 2.5 fields/s with a time-lapse videocassette recorder (VCR; Panasonic, AG-6720) and replayed at 60 fields/s to facilitate quantification. The average rate of rotation in each 10-min interval was analyzed by counting the total number of complete revolutions in 6–8 min. The total number of rotational surges (Diefenbach *et al.*, 1991) was similarly counted at 10-min intervals.

Results

Ex ovo *culture of decapsulated embryos*

Initial attempts to achieve sustained development *ex ovo* were made with stage E15, E20, and E25 embryos, corresponding to the period of development of the rotational behavior and underlying ENC1-ciliary neural circuits (Diefenbach *et al.*, 1991, 1998; Koss *et al.*, 2002). A variety of culture media were tested, including AHS, APW, HDM, 30% M199, 5% galactose, 1.5% CF, and albumen-gland-conditioned medium. Although short-term growth and development was observed in all the media tested, none of them supported sustained growth, probably due to the absence of crucial nutritive components or growth-promoting factors. Of all the media tested, 30% M199, 1.5% CF, and albumen-gland-conditioned medium produced the best development, with stage E15, E20, and E25 embryos sometimes reaching stages that display features of normal stage E25, E30, and E40 embryos, respectively, after 3 d of culture (Fig. 1). At the same time point, the control embryos developing *in ovo* consistently reached stages E35, E45, and E50, respectively, when egg masses were immersed in APW (Fig. 1). Similarly, *in ovo* development proceeded normally when egg masses were incubated in HS.

Generally, embryos cultured *ex ovo* displayed abnormal features (Fig. 1) and slower development (Fig. 2) in comparison to *in ovo* controls. In particular, shell formation was either stunted or absent in the *ex ovo* embryos (Fig. 1). Furthermore, accumulation of particles was always found in the canaliculus of the protonephridia (arrows in Fig. 1). Finally, embryos were often hydropic after 72 h of culture *ex ovo*. Although embryos often survived in culture for more than 30 days, no significant development was observed after the first 5 days of *ex ovo* cultivation (Fig. 2). The average increases in the length of embryos over this time period were 54 ± 13 (SE) μm (n = 7) and 69 ± 11 μm (n = 7) for the stage E15 and E20 groups, respectively. In contrast, control embryos exhibited significantly greater increases in length over the same time periods (Fig. 2).

To test whether the high viscosity or other physical properties within egg capsules are important factors promoting embryonic development, embryos were cultured in AHS containing 1% gelatin or in hanging droplets of AHS. Neither of these conditions improved *ex ovo* embryonic development (data not shown).

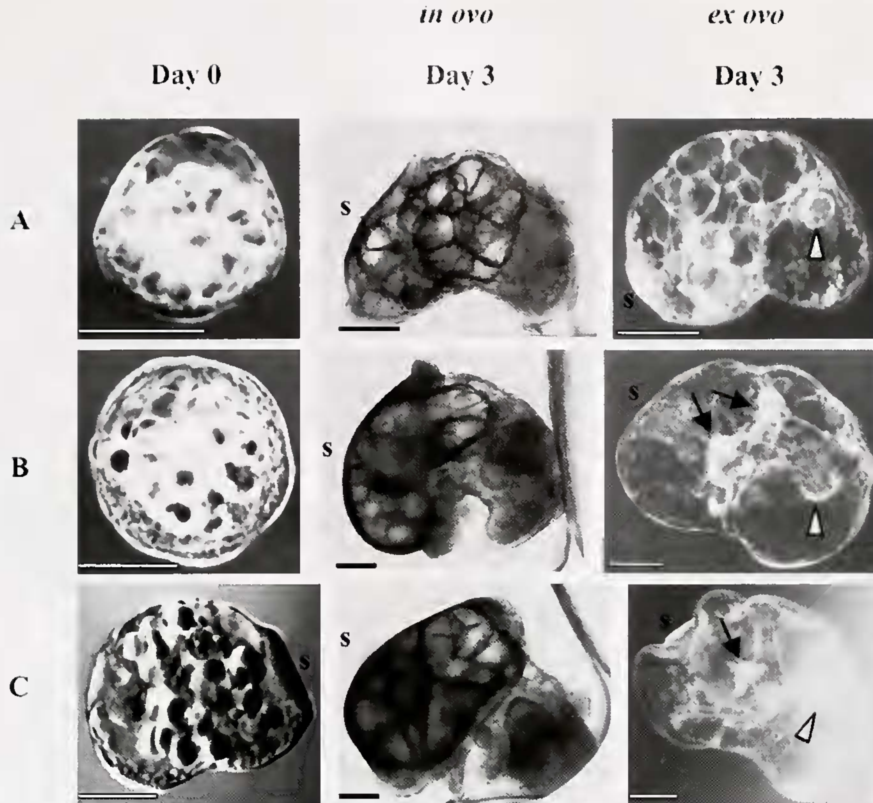

Figure 1. Embryonic development of *Helisoma* embryos after 3 days of culture *in ovo* and *ex ovo* at 23 °C. Examples of stage E15 (A), E20 (B), and E25 (C) embryos before culture (Day 0), after 3 days of culture in host egg capsules (*in ovo*), and in *Helisoma* saline plus 1.5% CF (*ex ovo*). Embryos cultured *in ovo* reached stages E40 (A), E45 (B), and E50 (C), whereas embryos cultured *ex ovo* only developed to stage E30 (A and B) or E35 (C). s: shell gland; arrowheads: radular sac; arrows: granular deposition in the protonephridium. Scale bars: 100 μm.

We next tested the effect of pH on the growth of isolated embryos. Egg mass matrix and CF had a pH of 6.6 ± 0.1 ($n = 4$), suggesting that low pH may promote embryonic development. The growth and development of stage E20 embryos were assessed after 3 days of culture in HDM adjusted to pH 6.4, 7.4, or 8.4. Although none of these groups exhibited significantly improved embryonic development as compared to the previous treatments, embryo development improved inversely with pH, both in terms of embryo length (Fig. 3A) and percent of embryos displaying stage E30 features, including larval kidney formation, muscle contraction of the body wall, and a shell (Fig. 3B). This effect of pH was not observed when embryos were cultured in 30% M199 (data not shown), a medium that generally promoted greater embryonic development than HDM, as described above.

Because a novel EGF has recently been identified in the CF-secreting albumen gland of *Lymnaea stagnalis* (Hermann *et al.*, 2000), we also tested whether EGF can promote the growth and development of *Helisoma* embryos *ex ovo*. Stage E20 embryos cultured for 3 days in HDM at pH 6.4 developed similarly, in both stage and body length, in the presence or absence of human recombinant EGF (450 ng/ml) or *Lymnaea* EGF (100 ng/ml) (data not shown). Although EGF may still be an important factor, other key components are clearly required to sustain normal growth and development of embryos *ex ovo*.

Transplantation of decapsulated embryos into host egg capsules

As an alternative to *ex ovo* culture of embryos, we tested the feasibility of transplanting decapsulated embryos into egg capsules already containing host *Helisoma* embryos. Stage E15 and E20 embryos were transplanted into host egg capsules containing much younger embryos (≤stage E10) to minimize mechanical interactions between host and transplant, and to ensure that they could be easily distinguished. Furthermore, younger egg capsules provide greater nutrition, since the nutritive components of CF become

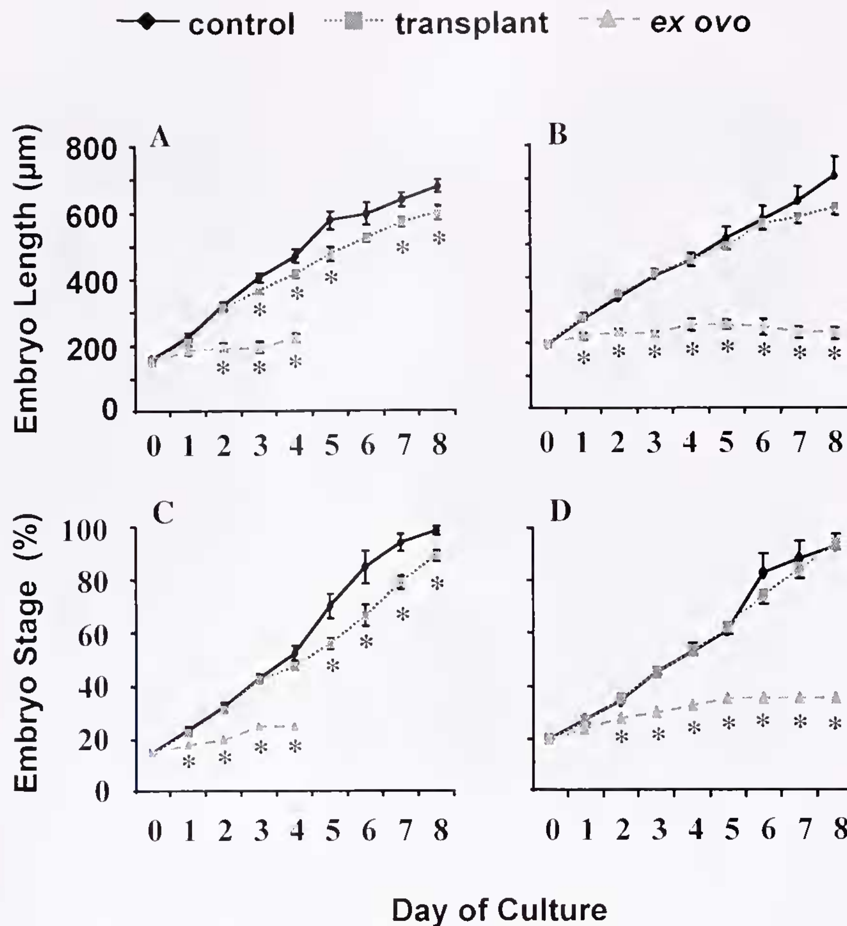

Figure 2. Growth and development of *in ovo* control embryos, transplanted embryos, and *ex ovo* (1.5% capsular fluid, pH 7.3–7.4) cultured embryos at 23 °C. The growth (length of embryos) of stage E15 (A) and E20 (B) embryos cultured *ex ovo* was significantly slower than that of control embryos (Student's *t* test, $P < 0.05$ at time points marked by asterisks, $n = 7$ for each of the *ex ovo* culture trials [10–15 embryos in each trial], $n = 6$ and 5 for control stage E15 and E20 embryos, respectively). In contrast, growth of transplanted stage E15 and E20 embryos was very similar to their control counterparts. Slight differences were seen only at later stages of culture in transplanted E15 embryos (marked by asterisks; *t* test, $P < 0.05$, $n = 6$). There was no significant difference in the growth of control and transplanted stage E20 embryos at any time points (*t* test, $P > 0.05$, $n = 6$). The development of the same stage E15 (C) and E20 (D) embryos cultured *ex ovo* was much slower than that of control embryos (asterisks; *t* test, $P < 0.05$). In transplanted stage E15 and E20 embryos, however, the development was very similar to their control counterparts. Small differences were only seen at later stages of culture in transplanted E15 embryos (asterisks; *t* test, $P < 0.05$). There was no significant difference in the development of control and transplanted stage E20 embryos at any time point (*t* test, $P > 0.05$).

depleted as embryonic development proceeds (Morrill, 1982; Stockmann-Bosbach and Althoff, 1989). In most cases, the incision in the capsule membrane re-sealed within 12 h after transplantation.

In striking contrast to the *ex ovo* trials, the growth and development of transplanted embryos was very similar to that displayed by control embryos within intact egg capsules (Fig. 2). At 23 °C, the growth and development of transplanted stage E15 embryos were identical to the controls in the first 3–4 days but slightly slower thereafter (Fig. 2A, C). When transplanted at stage E20 or older, however, the lengths (Fig. 2B) and embryonic stages (Fig. 2D) reached by transplanted embryos at various days were identical to controls (Student's *t* test, $P > 0.05$ at any time point, $n = 7$). Although embryos transplanted at stage E15 had a marginally lower survival rate 8 days after transplantation (Fig. 4A, chi square test, $P < 0.05$), the survival rate of embryos transplanted at stage E20 was not different from that of control (Fig. 4A, chi square test, $P > 0.05$). However, transplantation caused a slight decrease in the

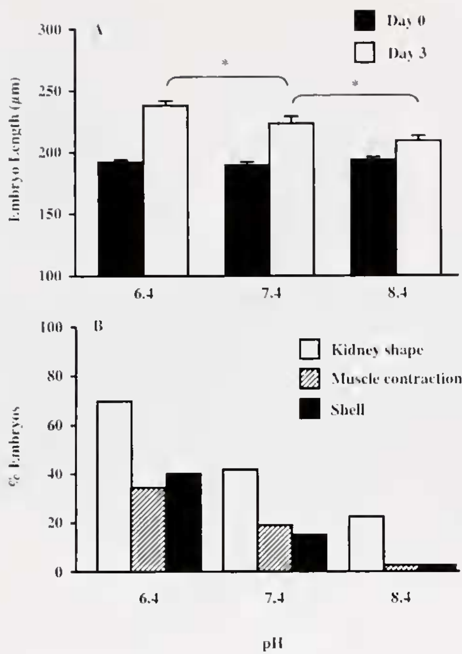

Figure 3. Effect of pH on growth and development of embryos cultured *ex ovo* in *Helisoma* defined medium at 23 °C. (A) Embryonic length before and after 3-day culture of stage E20 embryos. At each pH, there was a significant difference in the average embryonic length between Day 0 and Day 3 (Student's t test, $P < 0.05$, $n = 44$–66 embryos in 3 replicates). Whereas there were no differences in embryonic length at different pH at Day 0, there were significant differences in embryonic length at Day 3 (asterisks: t test, $P < 0.05$). (B) Percent of embryos expressing specific features of control stage E30 embryos, including larval kidney shape, muscle contraction of the body wall, and presence of a shell, at different pHs. The percentage expression of each feature was significantly different at these three pHs (G test, $P < 0.01$, $n = 51$–68 embryos from 3 replicates).

percentage of embryos hatched at 8 days post-transplantation (Fig. 4B); 91% stage E15 ($n = 23$) and 89% stage E20 ($n = 18$) control embryos hatched by this time, as compared to 70% stage E15 ($n = 28$) and 62% stage E20 ($n = 29$) transplanted embryos, respectively (chi square test, $P < 0.05$ for both stages). Nonetheless, transplanted embryos grew normally in APW after hatching (data not shown). These data suggest that transplantation into host egg capsules is an extremely effective technique for achieving a normal course of embryonic development for previously decapsulated stage E15 or older embryos.

To test whether this transplantation technique can be applied to embryos at cleavage stages, we transplanted 2-cell to 8-cell stage embryos (stage E1–E2) into capsules whose hosts were killed previously with heat shock (48 °C for 25 min). About two-thirds of the successfully transplanted embryos died at about stage E10, the time of gastrulation. However, the remaining third survived and displayed normal embryonic growth and development (Figs. 5 and 6).

An application of the embryo transplantation technique

Prior to development of the transplantation technique, we established the motor function of ENC1 by stimulating its release of serotonin with a laser treatment and recording the activity of postsynaptic ciliary cells (Kuang and Goldberg, 2001). It has been hypothesized that ENC1 regulates embryonic rotation through periodic release of serotonin to generate surges of embryonic rotation (Diefenbach *et al.*, 1991). Through transplantation, we can now provide further evidence in support of this hypothesis. Stage E25 embryos were removed from their egg capsules, subjected to bilateral laser ablation of ENC1s or neighboring control cells (Kuang and Goldberg, 2001), and transplanted into host egg capsules for behavioral analysis (Fig. 7A). After an overnight recovery period, the embryonic rotational rate and frequency of rotational surges were measured. In comparison to the control-ablated embryos, embryos with ENC1s ablated displayed a significant reduction in rotational rate (Fig. 7B, t test, $P < 0.05$, $n = 9$ for control and 7 for ENC1 ablation) and almost a total absence of rotational surges (Fig. 7C, t test, $P < 0.01$, $n = 11$ for control and 9 for ENC1 ablation). Therefore, the embryo transplantation technique can be instrumental in investigating cellular function in encapsulated embryos.

Discussion

An embryonic transplantation technique for long-term sustained growth and development of previously decapsulated embryos was reported in the present study. The *ex ovo* culture conditions that were also tested in this study were effective in the short-term maintenance of embryonic

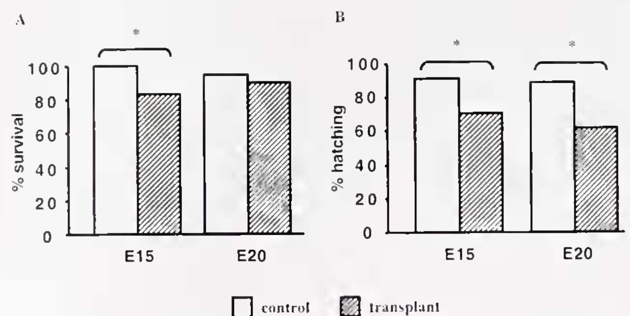

Figure 4. Survival and hatching rates of transplanted and control embryos at 23 °C. The survival rate was calculated as the total number of embryos alive at Day 8 as a percentage of total embryos at the beginning of the experiment. The hatching rate was calculated as the total number of embryos hatched as a percentage of the total embryos alive at Day 8. (A) Survival rate of control and transplanted stages E15 and E20 embryos. $n = 23$ (control E15), 30 (transplant E15), 19 (control E20), and 30 (transplant E20). Asterisk: $P < 0.05$, chi square test. (B) Hatching rate of control and transplanted stages E15 and E20 embryos. $n = 23$ (control E15), 28 (transplant E15), 18 (control E20), and 29 (transplant E20). Asterisks: $P < 0.05$, chi square test.

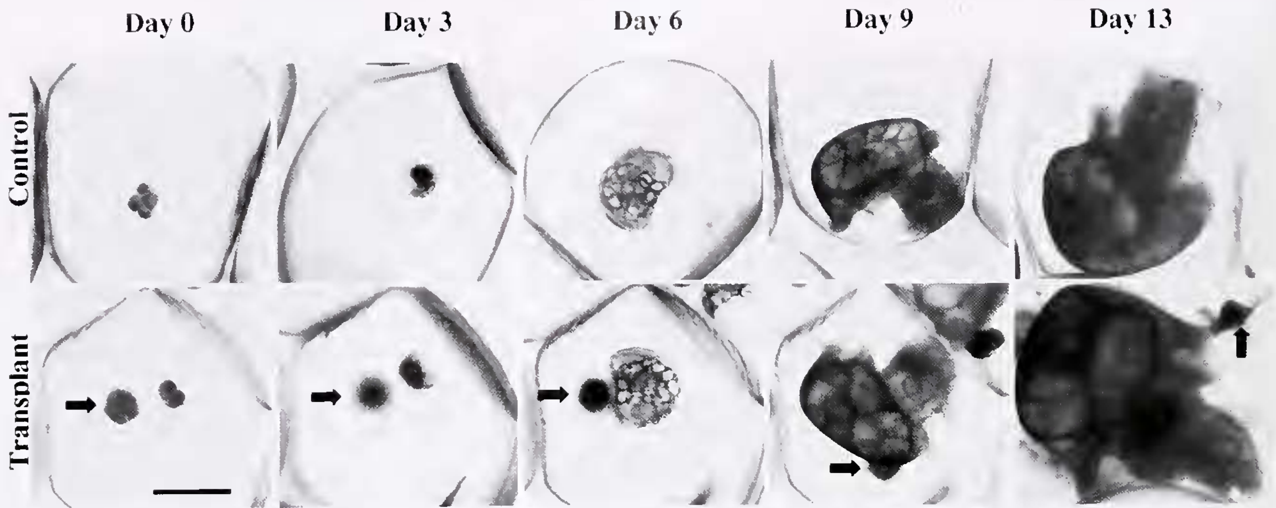

Figure 5. Morphological development of control and transplanted stage E1–E2 (2–4 cell stages) embryos at 20.5 °C. A control embryo developed from the 4-cell stage to hatching in 13 days. Similarly, a transplanted embryo developed from the 2-cell stage to hatching at the same rate. The transplanted embryo was isolated from the same egg mass as the control embryo. The transplanted embryos lagged behind the control embryo by 1 cleavage division at Day 0 because of the isolation and the 1-h incubation in saline prior to transplantation. The host embryo (arrows) in the transplantation group was killed by heat shock (45 °C, 25 min) prior to transplantation. Scale bar: 300 μm.

growth and survival, but failed to promote normal development over the long term.

That embryos at early stages (pre-shell stages) fail to develop normally under various *ex ovo* conditions suggests that some key growth-promoting factors or certain environmental conditions are missing in *ex ovo* culture. It has been reported in several species that encapsulated embryos cannot develop normally if they are removed from the egg capsules at early stages of development (Morrill, 1982; Pechenik *et al.,* 1984; Stockmann-Bosbach and Althoff, 1989; Meshcheryakov, 1990). This is probably because the environment in the capsular matrix is extremely complex and can hardly be mimicked *in vitro*. The situation is further complicated by the substantial variation in the composition of CF among species (Morrill *et al.*, 1964; Morrill, 1982; Heras *et al.*, 1998). Even less is known about the physical properties of the CF and the membranes surrounding it, such as viscosity, electrical charge, pressure, osmolarity, and pH (Beadle, 1969; Taylor, 1973; Morrill, 1982). For instance, this study indicates that *Helisoma* embryos naturally develop in a slightly acidic environment. Furthermore, acidic pH promoted growth and development of early embryos cultured *ex ovo* in *Helisoma* defined medium (HDM) (Fig. 3). Likewise, hamster embryonic cells exhibit optimal clonal proliferation when cultured in medium with a pH of 6.70 (Leboeuf *et al.*, 1989). We do not know why embryonic growth was not promoted by acidic pH when cultured in 30% M199. Intracapsular hydrostatic pressure may also be an important factor for normal embryonic growth and development. In *Lymnaea palustris*, early stage embryos

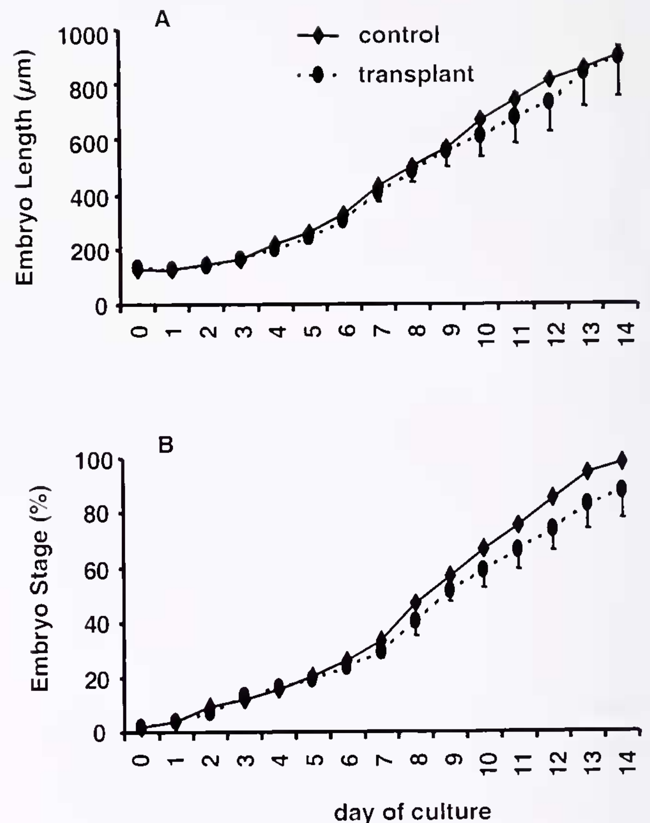

Figure 6. Growth (A) and development (B) of control and transplanted stage E1–E2 (2–8 cell stage) embryos at 20.5 °C. No difference in growth or development was found between control and transplanted embryos ($P > 0.05$ on each day, $n = 22$ and 4 for control and transplanted embryos, respectively).

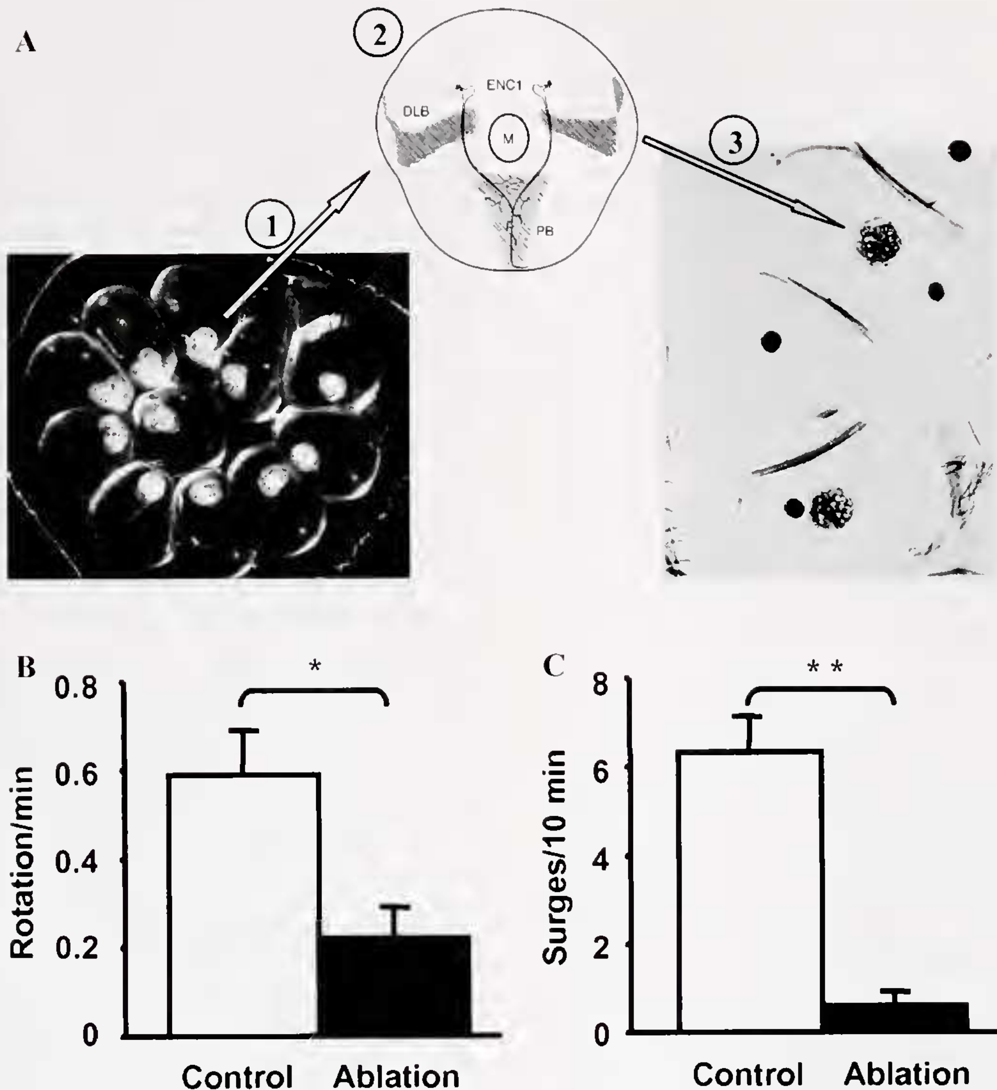

Figure 7. An application of embryo transplantation in studying embryonic cell function. (A) Diagram showing the experimental design. Stage E25 embryos were isolated from egg capsules① and a pair of embryonic neurons (ENC1s) or control cells were ablated with laser beams under a compound microscope②. The laser-operated embryos were transplanted into host egg capsules that contain much younger embryos③. The embryonic rotational behavior was subsequently examined after overnight recovery. DLB: dorsolateral ciliary band; M: mouth; PB: pedal ciliary band. (B) The average rate of rotation was reduced in embryos with both ENC1s ablated (*: t test, $P < 0.05$, $n = 9$ and 7 for control and ENC1 ablated embryos, respectively). (C) The occurrence of rotational surges was almost abolished in embryos with both ENC1s ablated (**: t test, $P < 0.01$, $n = 11$ and 7 for control and ENC1 ablated embryos, respectively).

fail to develop normally if their egg capsules are punctured at 2–4 h intervals to relieve the intracapsular hydrostatic pressure (Morrill, 1982). Pechenik *et al.* (1984) reported increased survival when decapsulated prosobranch embryos were cultured in a pressurized syringe, yet they all failed to develop normally.

Various kinds of abnormal development were observed when embryos were cultured *ex ovo*. In our study, embryos incubated in culture media always accumulated a large number of particles in the canaliculus of the protonephridia, suggesting either a malfunction of these embryonic kidneys or a precocious development of nitrogen catabolism (Morrill, 1982). In addition, all embryos in culture failed to grow a shell and a mantle, indicating incomplete development of the shell gland or lack of $CaCO_3$ in the media. Taylor (1973) reported that a considerable amount of Ca^{2+} in CF is bound to calcium buffer. Given the reasonably high Ca^{2+} concentration in the culture medium, the bound Ca^{2+} that normally

exists in the CF may be important for shell formation and development in general. Finally, *ex ovo* cultured embryos were sometimes hydropic after 72 h, suggesting that the osmoregulatory and extracellular fluid control machineries are impaired in these embryos, or that the osmolarity of the media is outside the range that embryos can handle.

Although *Helisoma* and other encapsulated pulmonates undergo direct development, they may still rely on chemical cues similar to those used by indirect developers to trigger settlement and metamorphosis (Morse, 1990). CF may contain cues that allow development to proceed from E30–E50, the stages resembling the transition from veliger to juvenile in metamorphosing gastropods (Raven, 1958; Morrill, 1982). That embryos develop normally when cultured after this transition lends support to this hypothesis (Pechenik *et al.*, 1984). Our preliminary trials also indicate that isolated stage E45 or later embryos grow and develop normally in artificial pond water (APW). Considering that epidermal growth factor (EGF) has recently been identified as a major component in the albumen gland and CF in *Lymnaea* and has been implicated in development of the nervous system (Hermann *et al.*, 2000), it may serve as a metamorphic factor in CF. *Lymnaea* EGF shares 35% of the amino sequence with the well-conserved mammalian EGFs, but lacks a transmembrane domain (Hermann *et al.*, 2000). However, neither human nor *Lymnaea* EGF promoted growth and development of *Helisoma* embryos. It is likely that EGF alone is not sufficient to compensate for other crucial factors that are missing in the culture media, or that the EGFs used in this study are highly species-specific.

If we ignore the physical properties of the intracapsular environment for the moment, CF intuitively seems to be the best medium for cultivation of decapsulated embryos. However, harvest of large quantities of concentrated CF is technically difficult because each capsule contains only 0.47 μl of the fluid. Moreover, when extracted from egg capsules, the highly viscous CF often clogged the micropipette or solidified in air. We have also tried to culture decapsulated embryos in the liquid fraction of egg mass homogenates, with no apparent improvement in embryonic development. The degradation of certain essential components by enzymes released from homogenized embryonic cells or the inability to mimic the physical environment of the intact egg capsule likely contributed to the poor development. Since the CF is secreted by albumen gland *in vivo*, a recent study showing that cAMP stimulates albumen gland secretion (Morishita *et al.*, 1998) provides a convenient way to produce CF *in vitro*. In future studies, albumen-gland-conditioned medium in combination with physical manipulations such as positive hydrostatic pressures should be attempted.

Transplantation of previously decapsulated gastrulated embryos yields a survival and growth rate similar to that of control embryos, suggesting that experimental manipulations and decapsulation of gastrulated embryos do not permanently hinder embryonic development. We observed that the ruptured openings on the capsular membranes of most egg capsules were self-sealed by 12 h after transplantation. Rich disulfide bonds previously reported in the egg covering of *Littorina saxatilis* (Losse and Greven, 1993) may have played a role in the self-sealing process. Alternatively, the phenomenon might result from the solidification of CF along the suture. In addition, certain physical or chemical properties of the gelatinous matrix surrounding the exterior of the egg capsule membranes may facilitate the sealing process. The layer of polyanionic acid mucopolysaccharide on the surface of capsular membrane (Plesch *et al.*, 1971) might be repelled by APW and therefore keep the ruptured membrane at a closely apposing state. The self-sealing of the ruptured capsular membrane is important in maintaining the physical, chemical, and electrical properties of the intracapsular environment that are essential for normal growth and development of embryos.

Roughly two-thirds of the embryos transplanted at the 2- or 4-cell stages died during gastrulation. Since the heat-treated host capsules resealed equally as well as normal capsules after transplantation, the high mortality of transplanted early stage embryos cannot be attributed to a loss of hydrostatic pressure within the host egg capsules. Instead, this high mortality indicates either that embryos are especially vulnerable to experimental manipulation at early cleavage stages, or that gastrulation is a crucial process during embryonic development. Early in development, each embryo is surrounded by a vitelline membrane that is cast off during gastrulation (Morrill, 1982). The vitelline membrane is very sticky, so that it tends to attach to petri dishes and glass pipettes during isolation and transplantation of embryos. Although we used Sigmacote-coated petri dishes and glass pipettes to prevent adhesion, it is still possible that the vitelline membranes of many embryos were ruptured during experimental manipulation. In *Lymnaea*, fertilized eggs whose vitelline membrane have been chemically removed can develop into embryos (Morrill, 1982); however, the long-term viability of these embryos is unknown. If the integrity of the vitelline membrane is crucial for survival of *Helisoma* embryos, its disruption may explain the high mortality of transplanted cleavage-stage embryos. Alternatively, it is possible that certain key "gastrulation factors" in the host CF are denatured or inactivated during the heat-shock treatment used to kill the host embryos before transplantation. Our preliminary experiments indicated that more cleavage-stage embryos survived gastrulation (80%, $n = 10$) when they were transplanted into host egg capsules that had not been heat shocked. It remains to be determined, however, whether egg capsules contain heat-labile gastrulation factors.

The transplantation technique developed in this study provides a unique opportunity to assess the long-term effects of experimental manipulations on encapsulated em-

bryos. For example, the loss of rotational surges after laser ablation of both ENC1s confirms that these cells function as cilioexcitatory motor neurons (Kuang and Goldberg, 2001). Through transplantation of ENC1-ablated embryos, we have recently demonstrated that ENC1s mediate oxygen sensing and an adaptive response to hypoxia (Kuang *et al.*, 2002). It is now possible to examine the long-term effect of ENC1 ablation on the survival of embryos, as well as on respiratory function after hatching. The embryonic transplantation technique will also be useful in examining cell interactions during neuronal differentiation, neurite outgrowth, and synaptogenesis *in vivo*. Given the organizational simplicity of the ENC1-cilia circuit in *Helisoma* embryos, it is feasible to look into the interaction between ENC1 and its target ciliary cells by ablating either of them and examining the developmental consequences. Finally, studies involving cell lineage tracing or genetic manipulations are now possible, since molecular markers or genetic materials can be injected into early embryos and these embryos can be transplanted into host egg capsules for further development. With the recent molecular cloning of serotonin receptors in *Helisoma* (Mapara *et al.*, 2001), it is possible to look at the developmental and physiological consequences of over- or under-expression of these receptors. These are a few examples of how the transplantation technique can facilitate the investigation of physiological and developmental events in embryos of *Helisoma* and other encapsulated species.

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